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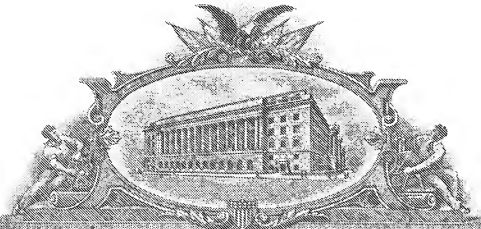

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APPLICATION NUMBER: ***60/549,487***
FILING DATE: ***March 01, 2004***
RELATED PCT APPLICATION NUMBER: ***PCT/US05/04868***

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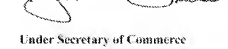

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV330728135US

22151 U.S. PTO 60549487 030104

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Raymond L.	Rodriguez	Davis, California
Mark Jesus M.	Magbanua	Davis, California

☐ *Additional inventors are being named on the____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

GENE EXPRESSION PROFILES OF NORMAL AND TUMORIGENIC CELLS

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages*	22	☐ CD(s), Number	
☒ Drawing(s) *Number of Sheets*	1	☒ Other (specify)	1. Fee Transmittal 2. Return Postcard
☐ Application Data Sheet. See 37 CFR 1.76			

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

		FILING FEE AMOUNT ($)
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☒	A check or money order is enclosed to cover the filing fees	
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____ Date 3-1-04

TYPED or PRINTED NAME Nan Wu REGISTRATION NO. 43,360 *(if appropriate)*

TELEPHONE (415) 836-2500 Docket Number: 693243-197 (UCD1210)

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FEE TRANSMITTAL for FY 2004

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☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) **$80.00**

Complete if Known	
Application Number	New
Filing Date	Herewith
First Named Inventor	Raymond L. Rodriguez
Examiner Name	N/A
Art Unit	N/A
Attorney Docket No.	693243-197 (UCD1210)

METHOD OF PAYMENT *(check all that apply)*

☒ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☐ Deposit Account:

Deposit Account Number: 07-1896

Deposit Account Name: Gray Cary Ware & Freidenrich

The Director is authorized to: *(check all that apply)*

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) **$80.00**

2. EXTRA CLAIM FEES FOR UTILITY AND

	Extra Claims		Fee from below	Fee Paid
Total Claims	-20** =	0	X	= 0.00
Independent Claims	- 3** =	0	X	= 0.00
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) **$0.00**

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non - English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR § 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Statement	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR § 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR § 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($)

SUBMITTED BY				Complete *(if applicable)*	
Name (Print/Type)	Nan Wu	*Registration No. (Attorney/Agent)*	43,360	*Telephone*	(415) 836-2500
Signature				*Date*	March 1, 2004

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CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10)			Docket No. 693243-197 (UCD1210)
Applicant(s): Raymond L. Rodriguez, et al.			
Serial No. New	Filing Date Herewith	Examiner N/A	Group Art Unit N/A

Invention:

GENE EXPRESSION PROFILES OF NORMAL AND TUMORIGENIC CELLS

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EV 330728135US
("Express Mail" Mailing Label Number)

Gene Expression Profiles of Normal and Tumorigenic Cells

This invention was made in part with government support under Grant No. P60MD00222 awarded by the National Institutes of Health (NIH). The government may have certain rights in this invention.

FIELD OF THE INVENTION

This invention relates generally to the field of neoplastic growth, especially gene expression profiles associated with cancer or tumor.

BACKGROUND OF THE INVENTION

Numerous epidemiological studies have shown that dietary factors play an important role in the etiology of different kinds of cancer (Greenwald et al., 2001). For example, soybean-rich diets are associated with lower cancer mortality rates, particularly for cancers of the colon, breast and prostate (Messina et al., 1994). Isoflavones and the Bowman-Birk protease inhibitor (BBI) are some of the many components in soybean believed to be responsible for suppressing carcinogenesis (Kennedy, 1995). Recently, lunasin, a small peptide found in soybean seeds, has shown promise as a chemopreventive agent(Galvez et al., 2001).

Lunasin is a 43 amino acid small subunit of a soybean 2S albumin. The carboxyl end of lunasin contains a chromatin-binding domain, a cell adhesion motif Arg-Gly-Asp (RGD) followed by eight Asp residues (Galvez and de Lumen, 1997; Galvez and de Lumen, 1999). The chromatin-binding domain consists of a 10-amino acid helical region homologous to a short conserved region found in other chromatin binding proteins (Aasland and Stewart, 1995). Mammalian studies provide evidence that lunasin may play a role in the cell cycle control (Galvez and de Lumen, 1999; Galvez et al., 2001; Jeong et al., 2002). For example, transfection of the lunasin gene into mammalian cells results in mitotic

arrest and subsequent cell death (Galvez and de Lumen, 1999). In addition, exogenous addition of chemically synthesized lunasin to mammalian cells demonstrates that lunasin (a) colocalizes with hypoacetylated chromatin; (b) preferentially binds deacetylated histone H4 *in vitro;* and (c) prevents histone H3 and H4 acetylation *in vivo* in the presence of a histone deacetylase inhibitor (Galvez et al., 2001). Recently, lunasin was isolated from barley and was reported to possess the same biological activity ascribed to chemically synthesized lunasin (Jeong et al., 2002).

While transfection of lunasin leads to cell death, lunasin peptide has been shown to have chemopreventive properties (Galvez et al., 2001). Significant suppression of chemical carcinogen-induced, e.g. 7,12-dimethylbenz[a]-anthracene (DMBA) and 3-methylcholanthrene (MCA), foci formation in C3H 10T1/2 mouse embryo fibroblast cells was observed when lunasin was added exogenously at nanomolar concentrations. In addition, topical application of lunasin inhibited skin tumorigenesis in female SENCAR mice. Lunasin peptide has also been shown to induce apoptosis in E1A-transfected C3H10T1/2 cells (Galvez et al., 2001) and suppress foci formation in E1A-transfected mouse fibroblast NIH 3T3 cells (Lam, et al., in press). E1A is a viral onco-protein that inactivates the Rb (retinoblastoma) tumor suppressor (Nevins, 1992). Furthermore, when C3H 10T1/2 and MCF-7 human breast cancer cells were treated with lunasin in the presence of the histone deacetylase inhibitor, sodium butyrate, a 10- to 95-fold reduction in acetylation of core histones H3 and H4 was observed (Galvez et al., 2001). The genome-wide reduction in core histone acetylation suggests an epigenetic mechanism of action for lunasin that can influence gene expression fundamental to carcinogenesis.

Prostate cancer is the most common non-dermatological carcinoma in the United State with an estimated 220,900 new cases and 28,900 deaths in 2003 (ACS, 2003). This type of cancer is the second leading cause of death among American men (www.cancer.org). Effects of anticancer agents on gene expression profiles of prostate cell lines using cDNA microarray analysis have been reported (Kudoh et al., 2000; Li et al., 2003; Zembutsu et al., 2003).

Microanalysis has been a useful tool to simultaneously determine changes in gene expression levels of tens of thousands of genes. In this study, microarray analysis was used to determine the changes in gene expression profiles of normal (RWPE-1) and malignant (RWPE-2) prostate epithelial cell lines after 24 hour exposure to synthetic lunasin. Results of the microarray experiments substantiate the chemopreventive property of lunasin and help elucidate its molecular mechanism.

SUMMARY OF THE FIGURE

Figure 1 shows the real-time reverse transcription-polymerase chain reaction analysis of selected genes in normal prostate epithelial cells (RWPE-1) and malignant prostate epithelial cells (RWPE-2) treated with 2 μM lunasin. HIFIA-hypoxia-inducible factor 1, alpha subunit; PRKARIA- protein kinase, cAMP-dependent, regulatory, type I, alpha; TOB1- transducer of ERBB2, 1; THSB1- thrombospondin 1

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, in part, on the discovery that various gene expression profiles are associated with neoplastic growth and prevention or treatment thereof.

Lunasin, a unique 43-amino acid soybean peptide, has been shown previously to suppress chemical carcinogenesis in mammalian cells and skin tumor formation in mice. It has also been shown to a) lunasin colocalize with hypoacetylated chromatin; b) bind preferentially to deacetylated histones H4 *in vitro*; and c) inhibit histone H3 and H4 acetylation *in vivo* in the presence of a histone deacetylase inhibitor. Acetylation and deacetylation of conserved histone N-terminal tails result in chromatin conformational changes that induce or suppress gene expression. Lunasin has been hypothesized to modulate changes in chromatin organization by modifying histone tails, thereby, affecting gene expression that leads to its chemopreventive effect. In this study, normal (RPWE-1) and malignant (RWPE-2) prostate epithelial cells, were treated with

chemically synthesized lunasin peptide for 24 hours. The effects of exogenous lunasin on the comprehensive gene expression profiles were examined using Affymetrix Human Genome U133A Arrays that can screen up to 22,215 genes. Results of the microarray analysis showed that a total of 123 genes had a greater than twofold change in expression. Of these genes, 121 genes were up-regulated in RPWE-1 normal cells and only two genes were up-regulated in RPWE-2 malignant epithelial cells after 24h exposure to the lunasin peptide. No genes were down-regulated in both cell lines. Those genes up-regulated in RPWE-1 cells include genes involved in the control of cell division, tumor suppression and cell death. The microarray data was validated by performing real-time reverse-transcription PCR on selected genes. We propose that lunasin prevents cancer in vitro and in small animal models by up-regulating the expression of genes that prevent the onset of the disease. Lunasin appears to have little effect on cells already transformed. These results confirm previous findings that showed lunasin to be more chemopreventive rather than therapeutic.

Lunasin, a novel and promising chemopreventive soybean peptide, has been demonstrated to prevent cell transformation and foci formation induced either by chemical carcinogens and or by oncogene transformation of mammalian cells (Galvez et al., 2001). A previous study of genes affected by lunasin showed that the lunasin increased by five fold, the p21/WAF1/Cip1 protein levels in cell transfected by the E1A oncogene but not in untransfected control cells (Lam et al., in press). It was suggested that p21/WAF1/Cip1, a potent and universal inhibitor of cyclin-dependent kinases (Gartel et al., 1996; Gartel and Tyner, 1999), is the prime candidate gene regulated by lunasin. However, the mechanism for how lunasin affects p21/WAF1/Cip1 regulation was not established. In contrast, microarray results did not show up-regulation of p21/WAF1/Cip1 transcript. Instead, the gene SP3, a transcriptional activator of p21/WAF1/Cip1 (Sowa et al., 1999) was up-regulated.

The molecular mechanism for how lunasin suppresses cell transformation is still unknown. A model to explain how lunasin suppresses E1A cell transformation has been proposed by Lam et al. (in press). The model proposes that in the presence of lunasin, inactivation of the Rb (retinoblastoma) by E1A dissociates the Rb-HDAC (histone deacetylase) complex and reveals deacetylated core histones. Lunasin competes with HAT in binding to the deacetylated core histones and keeps the chromatin in a condensed and transcriptionally inactive state. The cell perceives this condition as abnormal and commits itself to apoptosis.

Besides the Western blot analysis of p21/WAF1/Cip1 protein levels, nothing is known about what genes are regulated by lunasin. In this study, we performed a microarray determine the global gene expression profile of normal and malignant prostate epithelial cells after exogenous addition of lunasin peptide into the culture media. The microarray data reveal that lunasin up-regulates genes that are involved in mitotic check point, tumor suppressor genes and genes that are involved in apoptosis. This suggests that lunasin may be acting as a transcriptional activator that up-regulates genes that protect the cells from transformation. This interpretation is contrary to a previously proposed role of lunasin as a repressor of gene expression (Galvez et al., 2001). It is also interesting to note that lunasin does not have an effect on prostate epithelia cells that are already malignant. This finding agrees with previous experiments which show that lunasin does not appear to have an effect on a cell that is already transformed, at least, either by chemical carcinogens or by oncogene E1A (Galvez, unpublished data).

Aside from lunasin, several other components of soybean have been proposed to act as anti-cancer agents (Kennedy, 1995). For example, the Bowman-Birk Inhibitor (BBI), a soybean derived serine protease has been shown to posses anti-carcinogenic activity in both *in vitro* and *in vivo* systems (Kennedy, 1998). However, in contrast to our results, BBI and BBI concentrate (BBIC), a soybean concentrate enriched in BBI, has no effect on normal prostate epithelial

cells but inhibited the growth, invasion and clonogenic survival of prostate cancer cell lines (Kennedy and Wan, 2002). The precise mechanism(s) for the suppressive effects of BBI and BBIC is currently under investigation. Another possible chemopreventive agent from soy is genistein, a major isoflavone in soybeans. Genistein has been found to inhibit carcinogenesis both *in vitro* and *in vivo* (Barnes, 1995) and it is known to inhibit the activation of the nuclear transcription factor, NF-κB and the Akt signaling pathway, both of which are known to maintain the balance between cell survival and apoptosis (Sarkar and Li, 2002). Evidence shows that genistein induces apoptosis by up-regulating Bax, a protein that antagonizes the anti-apoptotic function of Bcl-2 (Sarkar and Li, 2002). A recent study was performed to examine the gene expression profiles of PC3 prostate cancer cells treated with genistein (Li and Sarkar, 2002; Li and Sarkar, 2002). Results of the microarray shows that genistein regulates the expression of genes that are important to the control of cell growth, cell cycle, apoptosis, cell signaling, angiogenesis, tumor cell invasion and metastasis (Li and Sarkar, 2002; Li and Sarkar, 2002). The gene expression data revealed possible molecular mechanisms by which genistein exerts its inhibitory effects on prostate cancer cells.

This study is the first to use gene expression profiling to investigate the potential chemopreventive properties of a peptide derived from soybean. Based on the data obtained from our microarray analysis, we propose that lunasin may be a transcription activator that up-regulates genes necessary for guarding or protecting the cells from transformation events. We believe lunasin may prime the cell for apoptosis and up-regulates the expression of some tumor suppressor genes and genes involved in the activation of mitotic checkpoint. Furthermore, the selective effect of lunasin on human gene expression supports an extensive body of epidemiological data linking high soybean intake and reduced risks of certain types of cancer such as breast, prostate and colon (Messina et al., 1994)

Because of its chemopreventive properties, we propose that lunasin may serve as a biopharmaceutical against cancer.

Lunasin, or variants thereof, can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the active ingredient and a pharmaceutically acceptable carrier. As used herein, the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds also can be incorporated into the compositions.

For the purposes of the instant invention, a variant is a lunasin molecule that includes one or more amino acid mutations in the native sequence. The mutation can be an insertion, deletion or substitution. Variants can be made using any of a variety of methods well known in the art. The changes can be made at any site of lunasin and performance of mutation at any one site can be optimized. Mutants are screened for the optimal combination of at the least desired neoplastic prophylactic activity, revealed for example, by the microarray screening assay disclosed herein. Thus, a suitable variant is one when using the Affymetrix Human Genome U133A array, would activate at least particular genes of interest, such as PKA, TOB1, ERBIN, NIP3, TSP1, BUB1B, TTK, PSMC6, USP1 and the like, in normal cells, or would activate a minimum number of genes or markers, such as at least 25 genes or markers, at least 50 genes or markers, at least 75 genes or markers and so on, in a non-malignant cell as compared to the number of genes activated in a paired malignant cell, as taught herein.

A pharmaceutical composition of the invention for use as disclosed herein is formulated to be compatible with the intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal and rectal administration. Solutions or suspensions used for parenteral, intradermal or subcutaneous application can include the following

components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as HCl or NaOH. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL® (BASF; Parsippany, NJ) or phosphate-buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. The composition must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol and the like) and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze drying that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. The composition can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches or capsules. Oral compositions also can be prepared using a fluid carrier to yield a syrup or liquid formulation, or for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed.

Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate or orange flavoring.

For administration by inhalation, the compound is delivered in the form of, for example, an aerosol spray from a pressurized container or dispenser that contains a suitable propellant, e.g., a gas such as carbon dioxide or a nebulizer, or a mist.

Systemic administration also can be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants generally are known in the art and include, for example, for transmucosal administration, detergents, bile salts and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels or creams as generally known in the art.

The compound also can be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compound is prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters and polylactic acid.

Methods for preparation of such formulations will be apparent to those skilled in the art. The materials also can be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc.

Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies) also can be used as pharmaceutically acceptable carriers. Those can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The dosages, for example, preferred route of administration and amounts, are obtainable based on

empirical data obtained from preclinical and clinical studies, practicing methods known in the art. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of the therapy is monitored easily by conventional techniques and assays. An exemplary dosing regimen is disclosed in WO 94/04188. The specification for the dosage unit forms of the invention is dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The pharmaceutical compositions can be included in a container, pack or dispenser together with instructions for administration.

Another method of administration comprises the addition of a compound of interest into or with a food or drink, as a food supplement or additive, or as a dosage form taken on a prophylactic basis, similar to a vitamin. The peptide of interest can be encapsulated into forms that will survive passage through the gastric environment. Such forms are commonly known as enteric-coated formulations. Alternatively, the peptide of interest can be modified to enhance half-life, such as chemical modification of the peptide bonds, to ensure stability for oral administration, as known in the art.

The instant invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) cancer.

In one aspect, the invention provides a method for preventing in a subject, a cancer by administering to the subject lunasin or a variant thereof. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of a cancer, such that a cancer disease or disorder is prevented. Alternatively, if symptomology exists, lunasin or a variant thereof can be administered to delay disease progression.

The invention now will be exemplified in part by the following non-limiting examples.

Examples

Cell culture and lunasin treatment. Prostate epithelial cells, RWPE-1 (normal) and RWPE-2 (malignant) were cultivated in the Keratinocyte-Serum free medium with 5ng/ml rEGF and 0.05 mg/ml bovine pituitary extract. Cells were grown to 70% confluence, after which, cells were harvested and transferred to a 150 mm^2 plate at a cell density of 1×10^7 cells/ml. Lunasin peptide was added to a final concentration of 2μM. The cells were incubated for 24 hours after lunasin treatment. Lunasin has been shown to localize in the nucleus after 18 hours of incubation (Yi et al. in press). RWPE-1 and RWPE-2 cells that were not treated with lunasin served as negative controls.

Microarrray analysis. Total RNA from each treatment was isolated by Trizol (Invitrogen, Carlsbad, CA). The cDNA used in microarray analysis was synthesized from 10 μg of total RNA using the SuperScript Choice system (Invitrogen). The cDNA was then transcribed *in vitro* in the presence of biotin-labeled nucleotide triphophates using T7 RNA polymerase after phenol-chloroform extraction and ethanol precipitation. cRNA was purified using the RNeasy mini kit (Qiagen) and fragmented at 94°C for 30 min in the buffer containing 0.2 M Tris-acetate (pH 8.1) 0.5 M potassium acetate, and 0.15 M magnesium acetate. Fragmented cRNA was hybridized overnight at 45°C to the human genome U133A Genechips (Affymetrix) representing approximately 22,215 transcripts. Hybridization was then detected using a confocal laser scanner (Affymetrix). The gene expression levels of samples were normalized and analyzed using RMA analysis (Irizarry et al., 2003). RMA analysis has been shown to have a higher sensitivity and specificity than dCHIP (Li and Wong, 2001) or MAS 5.0 (Affymetrix, 1999). Average-linkage hierarchal clustering of

data was applied using the Cluster (Eisen et al., 1998) and the results were displayed with TreeView (Eisen et al., 1998).

Quantitative PCR. To confirm microarray data, the total RNA prepared for microarray analysis was used for RT-PCR analysis of selected genes. The cDNA used for quantitative PCR was synthesized from 3 µg of total RNA using the SuperScript First-Strand Synthesis for RT-PCR kit (Invitrogen), following manufacturer's recommendations. The cDNA was diluted 4-fold and 2µl of cDNA was added to the quantitative PCR reaction using FAM-labeled TaqMan probes purchased from Applied Biosystems. The quantitative PCR reactions were performed in triplicate using a PRISM® ABI 7900HT Sequence Detection System (Applied Biosystems) and the expression of the ß-actin gene (BACT) was used for normalization. Changes in expression were calculated using relative quantification as follows: $\Delta\Delta Ct = \Delta Ctq - \Delta Ctcb$; where Ct = the cycle number at which amplification rises above the background threshold, ΔCt = the change in Ct between two test samples, q = the target gene, and cb = the calibrator gene. Gene expression is then calculated as $2^{-\Delta\Delta Ct}$ (Applied Biosystems).

Gene expression profiles.

The gene expression profiles of normal (RWPE-1) and malignant (RWPE-2) cells treated with lunasin were assessed using microarray analysis. The results of this analysis indicated that of the 22,215 gene interrogated, 123 genes had a greater than twofold change in expression. Of these genes, 121 genes were up-regulated in RPWE-1 cells and only two genes were up-regulated in RPWE-2 cells. No genes were down-regulated in either normal or malignant epithelial cells. Genes that were up-regulated in RPWE-1 cells include genes that are involved in the control of cell division, tumor suppression and cell death (Table 1).

Table 1. Partial list of genes up-regulated in normal epithelial cells (RWPE-1) after 24 hour exposure to 2 uM lunasin.

	Unigene cluster	Gene ID	Symbol	Fold-change
a) Tumor suppressive (anti-cell proliferation) genes				
protein kinase, cAMP-dependent, regulatory, type I, alpha	Hs.280342	5573	PRKAR1A, PKA	2.45
transducer of ERBB2, 1	Hs.178137	10140	TOB1	2.32
erbb2 interacting protein	Hs.8117	55914	ERBB2IP, ERBIN	2.23
b) Genes involved in apoptosis				
protein kinase C-like 2	Hs.69171	5586	PRKCL2, PRK2	2.33
BCL2/adenovirus E1B 19kDa interacting protein 3	Hs.79428	664	BNIP3, NIP3	2.05
thrombospondin 1	Hs.164226	7057	THBS1, TSP1	2.05
pro-oncosis receptor inducing membrane injury gene	Hs.172089	114908	PORIMIN	2.26
serine palmitoyltransferase, long chain base subunit 1	Hs.90458	10558	SPTLC1	2.12
c) Mitotic checkpoint control genes				
BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	Hs.36708	701	BUB1B, BUBR1	2.23
TTK protein kinase	Hs.169840	7272	TTK	2.08
MAD2 mitotic arrest deficient-like 1 (yeast)	Hs.79078	4085	MAD2L1	2.12
d) Protein degradation genes				
proteasome (prosome, macropain) 26S subunit, ATPase, 6	Hs.156171	5706	PSMC6	2.58
RAN binding protein 2	Hs.199179	5903	RANBP2	2.36
E3 ubiquitin ligase SMURF2	Hs.438968	64750	SMURF2	2.29
ubiquitin specific protease 1	Hs.35086	7398	USP1	2.23
ubiquitin-activating enzyme E1C (UBA3 homolog, yeast)	Hs.154320	9039	UBE1C	2.15

Lunasin upregulates genes for apoptosis

Transfection of lunasin into mammalian cells results in arrest of mitosis leading to apoptosis. The anti-mitotic effect of lunasin is attributed to the binding of its poly-aspartyl carboxyl end to regions of hypoacetylated chromatin, like that found in kinetochores in centromeres (Galvez and de Lumen, 1999). Apoptosis is thought to be triggered when the kinetochore complex does not form properly and the microtubules fail to attach to the centromeres that lead to mitotic arrest and eventually cell death. Results of the microarray analysis showed that exogenous addition of lunasin up-regulates certain genes that are known to have a direct or indirect role in the induction of apoptosis in cells. These genes include thrombospondin 1 (THBS1), BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), and protein kinase C-like 2 (PRKCL2). THBS1 (also called TSP-1) is a member of a family of extracellular proteins that participate in cell-to-cell and cell-to-matrix communication (Bornstein, 1995). The role of THBS1 in the process of apoptosis or programmed cell death in cancer cells has recently been reviewed (Friedl et al., 2002). THBS1 induces apoptosis by activating the caspase death pathway (Nor et al., 2000). In addition, THBS1 has also been shown to have a potent anti-angiogenic activity (Nor et al., 2000) and induction of apoptosis by THSB1 is associated with decreased expression of the anti-apoptotic protein, Bcl-2 (Nor et al., 2000). We find it intriguing that lunasin up-regulates the expression of BNIP3, a gene that inhibits the anti-apoptotic activities of Bcl-2. BNIP3, formerly known as Nip3, is a mitochondrial protein that activates apoptosis and overcomes Bcl-2 (anti-apoptotic protein), suppression of cell death (Chen et al., 1997; Chen et al., 1999; Ray et al., 2000). A more recent study shows that BNIP3 mediates a necrosis-like cell death independent of apoptotic events, such as release of cytochrome c, caspase activation and nuclear translocation of apoptosis-inducing factor (Velde et al., 2000). Evidence indicates that BNIP3 causes cell death through opening of the mitochondrial permeability transition pore resulting in mitochondrial dysfunction and plasma membrane damage. Another gene that is up-regulated by lunasin is the hypoxia-inducible factor-1α (HIF1-A), a basic-helix-loop-helix transcription factor which

regulates the expression of BNIP3 (Wang et al., 1995). The BNIP3 promoter contains a functional HIF-1-responsive element and is potently activated by both hypoxia and forced expression of HIF-1A (Bruick, 2000). Studies have also shown that HIF-1A binds and stabilizes p53, a tumor suppressor (An et al., 1998; Chen et al., 2003). On the other hand, PRKCL2, also termed PRK2, promotes apoptosis by inhibiting the anti-apoptotic activities of Akt, an oncogene (Koh et al., 2000). Akt exerts its anti-apoptotic effects by phosphorylation and thereby inactivating BAD, a pro-apoptotic Bcl-2 family protein (Khwaja, 1999). However, a PRKCL2 C-terminal fragment generated during the early stages of apoptosis binds Akt, resulting in the inhibition of the Akt-mediated phosphorylation of BAD, thereby allowing apoptosis to occur (Koh et al., 2000). Based on this microarray data, we propose that lunasin primes the cell for apoptosis, by upregulating genes that inhibit anti-apoptotic activities of Akt and some BCL2 members, e.g. BAD and BCL-2. We also suggest that lunasin may be indirectly involved in stabilization of tumor suppressor p53 via HIF1A.

Lunasin up-regulates genes involved in suppression of cell proliferation

Lunasin inhibitory effects on cell growth can be explained by the up-regulation of genes that play a role in tumor-suppression or anti-proliferation. For example, lunasin up-regulates a tumor suppressor gene encoding the cyclic AMP-dependent protein kinase A type I-α regulatory subunit, PRKARIA, (Sandrini et al., 2002). Mutations in the PRKARIA results in the Carney complex (CNC), a multiple neoplasia syndrome that is associated with thyroid tumorigenesis (Sandrini et al., 2002; Stergiopoulos and Stratakis, 2003). It is proposed that *PRKARIA* mutant cells have de-regulated control of gene expression which results in the activation of pathways, e.g. cAMP signaling, that lead to abnormal growth and proliferation (Stergiopoulos and Stratakis, 2003). Another gene up-regulated by lunasin, BTB (POZ) containing domain 1 (ABTB1 or BPOZ) is thought to be as one of the mediators of the growth-suppressive signaling pathway of the tumor suppressor, PTEN (Unoki and Nakamura, 2001). Overexpression of BPOZ inhibited cell cycle progression and suppressed growth

of cancer cells while the transfection of BPOZ anti-sense accelerates cell growth (Unoki and Nakamura, 2001). Tob (also referred to as Tob1) is up-regulated by lunasin and is a member of the anti-proliferative BTG/Tob family (Sasajima et al., 2002). Mice that are Tob deficient are prone to spontaneous formation of tumors (Yoshida et al., 2003). Further experiments also show that Tob acts a transcriptional co-repressor and suppresses the cyclin D1 promoter activity through an interaction with histone deacetylase (Yoshida et al., 2003). Our microarray results however, do not show down regulation of cyclin D1. The gene, erbb2 interacting protein (ERBIN), a novel suppressor of Ras signaling is up-regulated by lunasin. Erbin, a leucine-rich repeat containing protein, interacts with Ras and interferes with the interaction between Ras and Raf resulting in the negative regulation of Ras-mediated activation of extra-cellular signal regulated kinases (Erk) (Huang et al., 2003). The Ras oncogene is one of the most common mutations occurring in about 30% of human cancers (Duursma and Agami, 2003). Mutations that cause constitutive activation of Ras results in a continuous signal that tells the cells to grow regardless of whether or not receptors on the cell surface are activated by growth factor (Macaluso et al., 2002).

Lunasin up-regulates mitotic check point genes

Other genes up-regulated by lunasin is include the mitotic checkpoint genes like budding uninhibited benzimidazoles 1 homolog beta (BUB1B or BubR1), TTK protein kinase (MPS1 yeast homolog) and mitotic arrest deficient 2-like 1 (MAD2L1). Mitotic spindle checkpoint proteins monitor proper microtubule attachment to chromosomes prior to progression through mitosis allowing correct segregation of chromosomes into progeny cells (Lengauer et al., 1998). TTK is a protein kinase that phosphorylates MAD1p, a phosphorylation essential to the activation of the mitotic checkpoint (Farr and Hoyt, 1998). In yeast, MPS1 is required early in the spindle assembly checkpoint (Hardwick et al., 1996). It is suggested to be a limiting step in checkpoint activation, since it can activate the pathway when over expressed. Overexpression of MPS1 is able to delay cell

cycle progression into anaphase in a manner similar to check activation by spindle damage (Hardwick et al., 1996). MPS1 is also required of the essential process of spindle pole body duplication (Winey et al., 1991). BUBR1 is a protein kinase required for checkpoint control. Evidence shows that inactivation of BubR1 by microinjection of specific antibodies abolishes the checkpoint control (Chan et al., 1999). A study further revealed that endogenous BubR1 protein levels is decreased in some breast cancer cell lines; furthermore, evidence show, an oncogenic protein, breast cancer-specific gene 1 (BCSG1) directly interact is BubR1 which allows the degradation of BubR1 though a proteosome machinery (Gupta et al., 2003). It is speculated that BSCG1-induced reduction of the BubR1 protein allow breast cancer to progress at least in part by compromising the mitotic check point control through the inactivation of BubR1 (Gupta et al., 2003). Another check point gene MAD2L1 was reported to have reduced expression in a human breast cancer cell line exhibiting chromosome instability and aneuploidy (Li and Benezra, 1996). Some breast cancer cell lines show a mutation in the MAD2L1 gene that creates a truncated protein product; however, the specific role of MAD2L1 in breast cancer is still under investigation (Percy et al., 2000). We propose that lunasin up-regulates these mitotic check point genes to allow a heightened level of molecular surveillance to prevent premature cell division, chromosome instability and aneuploidy.

Lunasin upregulates genes involved in protein degradation

It is also interesting that lunasin up-regulates several genes involved in protein degradation and turnover via the ubiquitin pathway. These genes include the proteosome 26S subunit ATPase 6 (PSMC6); E3 ubiquitin ligase (SMURF2), ubiquitin specific protease 1 (USP1); and ubiquitin-activating enzyme E1C (UBE1C). It is possible that lunasin up-regulates these genes to mediate the degradation of proteins that are required for the onset of cell transformation and foci formation.

Lunasin up-regulates the gap junction protein, connexin 43

Adjacent cells communicate with each other through gap junctional channels that allow the passage of small molecules (Loewenstein and Rose, 1992). This process is referred to as "gap junctional intercellular communication" (GJC) and it is blocked in many cancer cells, including malignant human prostate cells (Hossain et al., 1999). Gap junctional channels are composed of proteins called connexins (Bruzzone et al., 1996). Lunasin up-regulates the expression of a gap junction protein called connexin 43, which has been shown to have a tumor suppressive role. Decreased expression and impaired posttranslational modification of connexin43 was observed in several prostate tumor cell lines but not in normal cells suggesting that the loss of junctional communication is a critical step in the progression to human prostate cancer (Hossain et al., 1999). Studies have shown that the viral oncogene Src disrupts cell growth regulation by adding a phosphate group to a tyrosine residue in connexin 43, thereby blocking gap junction communication (Kanemitsu et al., 1997). Transfection of a functional connexin 43 gene to tumorigenic mouse cells resulted in the restoration of GJC, normal growth regulation and cell-to-cell communication, as well as, suppression of tumorigenesis (Rose et al., 1993).

Target verification using RT-PCR.

Changes in mRNA level detected by microarray analysis were confirmed using real-time RT-PCR analysis of four genes: thrombospondin 1(THBS1), protein kinase, cAMP-dependent , regulatory, type 1 alpha (PRKAR1A), transducer of ERBB2, 1 (TOB1) and hypoxia inducible factor 1, alpha subunit (HIF1A) . The results of the real-time RT-PCR analysis for these selected genes were consistent with the microarray data. These results support our interpretation of the microarray data; that lunasin up-regulates the expression of genes of normal prostate epithelial cell but not in established malignant prostate epithelial cells.

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A method of preventing neoplastic growth in a cell comprising administering to the cell an agent, wherein the agent up-regulates the activity or expression of at least one, two, three, four, five, or six genes listed in Table 1.

2. A method of preventing neoplastic growth in a subject comprising administering to the subject an agent, wherein the agent up-regulates the activity or expression of at least one, two, three, four, five, or six genes listed in Table 1.

3. A method of preventing neoplastic growth in a cell comprising up-regulating the activity or expression of at least one, two, three, four, five, or six genes listed in Table 1.

4. A method of preventing neoplastic growth in a subject comprising up-regulating the activity or expression of at least one, two, three, four, five, or six genes listed in Table 1.

GENE EXPRESSION PROFILES OF NORMAL AND TUMORIGENIC CELLS

Abstract of the Disclosure

The present invention provides methods and agents useful for the prevention of neoplastic growth.

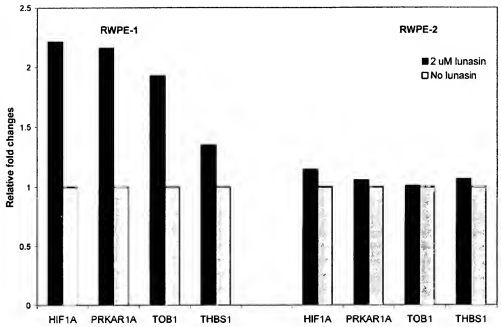
RWPE-1
RWPE-2
2 uM lunasin
No lunasin
Relative fold changes
2.5
2
1.5
1
0.5
0
HIF1A
PRKAR1A
TOB1
THBS1
HIF1A
PRKAR1A
TOB1
THBS1

Figure 1